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ORIGINAL PAPER

Isolation and characterization of halophilic Archaea able to produce biosurfactants

S. Kebbouche-Gana · M. L. Gana · S. Khemili · F. Fazouane-Naimi · N. A. Bouanane · M. Penninckx · H. Hacene

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Abstract Halotolerant microorganisms able to live in saline environments offer a multitude of actual or potential applications in various fields of biotechnology. This is why some strains of Halobacteria from an Algerian culture collection were screened for biosurfactant production in a standard medium using the qualitative drop-collapse test and emulsification activity assay. Five of the Halobacteria strains reduced the growth medium surface tension below 40 mN m^{-1}, and two of them exhibited high emulsion-stabilizing capacity. Diesel oil-in-water emulsions were stabilized over a broad range of conditions, from pH 2 to 11, with up to 35% sodium chloride or up to 25% ethanol in the aqueous phase. Emulsions were stable to three cycles of freezing and thawing. The components of the biosurfactant were determined; it contained sugar, protein and lipid. The two Halobacteria strains with enhanced biosurfactant producers, designated strain A21 and strain D21, were selected to identify by phenotypic, biochemical characteristics and by partial 16S rRNA gene sequencing. The strains have Mg^{2+}, and salt growth requirements are always above 15% (w/v) salts with an optimal concentration of 15–25%. Analyses of partial 16S rRNA gene sequences of the two strains suggested that they were halophiles belonging to genera of the family Halobacteriaceae, *Halovivax* (strain A21) and *Haloarcula* (strain D21). To our knowledge, this is the first report of biosurfactant production at such a high salt concentration.

Keywords Halobacteria · Screening · Biosurfactant · Surface tension

S. Kebbouche-Gana (✉) · N. A. Bouanane · H. Hacene
Faculty of Biological Sciences, Laboratory of Microbiology, U.S.T.H.B, Algiers, Algeria
e-mail: kebbouchesalima@yahoo.fr

S. Kebbouche-Gana · S. Khemili · F. Fazouane-Naimi
Department of Biology, University M'Hamed Bougara of Boumerdes, 1st November Avenue, 35000 Boumerdes, Algeria

M. L. Gana
Centre of Research and Development, Laboratory of Corrosion, SONATRACH, Avenue of 1st November, 35000 Boumerdes, Algeria

M. Penninckx
Service of Physiology and Microbial Ecology, England Avenue 642, 11180N Brussels, Belgium

Introduction

Biosurfactants are a diverse group of surface-active agents produced by many living organisms [6, 43]. These amphiphilic compounds contain a hydrophobic and a hydrophilic moiety, and have the ability to reduce interfacial tension between different fluid phases. Their uses and potential commercial applications have been reported in several fields, including surfactant-assisted flooding for enhanced oil recovery in the oil industry, emulsifiers in the food industry and moisturizers in the cosmetic industry [7, 13, 14, 22]. Biosurfactants are known to occur in a variety of chemical structures, such as glycolipids, lipopeptides and lipoproteins, fatty acids, neutral lipids, phospholipids, and polymeric and particulate structures [13].

The search for biosurfactants in extremophiles seems to be particularly promising since the biosurfactants of these organisms have particular adaptations to increase stability in adverse environments that can potentially increase their stability in the harsh environments in which they are to be applied in biotechnology [27, 53]. Some microorganisms

can survive and grow over a wide range of salt concentrations. In aquatic environments the conditions range from fresh water (containing less than 0.05% w/v dissolved salts), through sea water with total salinities of 3.2–3.8% (w/v) to saturated salt solutions up to 30% (w/v) and above [11, 40].

There are very few reports on biosurfactant producers in hypersaline environments [12, 36]. Halophiles, which have a unique lipid composition (phytanylglycerol), may have an important role to play as surface-active agents. The archae bacterial ether-linked phytanyl membrane lipid of the extremely halophilic bacteria has been shown to have surfactant properties [52]. Yakimov et al. [60] reported the production of biosurfactant by a halotolerant *Bacillus* species and its potential in enhanced oil recovery; *Bacillus licheniformis* strain BAS 50 was able to grow and produce a lipopeptide surfactant when cultured on a variety of substrates at salinities up to 13% NaCl. The production of bioemulsifiers from *Methanobacterium thermoautotrophicum* has been reported [56]. These bioemulsifiers were active over a wide range of pH (5–10) and at very high salt concentrations (up to 200 g l^{-1}). Recently, interest in the mass cultivation of microorganisms from hypersaline environments has grown considerably, because this represents an innovative low technology approach to biotechnological exploitation [45, 57]. In a screening program to obtain a biosurfactant producer, we have isolated obligately halophilic microorganisms from a previously unexplored site in Algeria. Hypersaline environments where salinities exceed 1.5 M are usually dominated by prokaryotes. Two main groups are to be found: the moderately halophilic bacteria are more abundant at intermediate salinities (1.5–2.5 M), whereas the halophilic Archaea (the halobacteria) dominate at salinities greater than 2.5 M, often imparting spectacular red pigmentation to the environment because of high levels of carotenoids [29]. Representatives of the majority of archaeal genera are characteristic of neutral saline environments (*Halobacterium*, *Halorubrum*, *Haloarcula*, *Haloferax*, *Halococcus*, *Halobaculum* and *Natrialba* spp) [29, 37, 38, 42, 46, 50], whereas alkaline saline environments harbor haloalkaliphilic halobacteria, such as *Natronomonas* and *Natronobacterium* spp [38].

The application of molecular and biochemical techniques has indicated that specific successions of halobacteria occur in hypersaline waters as the waters become concentrated [49]. Many neutral hypersaline environments at the saturation point harbor climax populations of halobacteria usually belonging to the genera *Halobacterium*, *Haloarcula* and *Halorubrum* [49]. Representatives of other genera are much less common [30, 54], although detailed characterization at the species level is seldom carried out. In this article we report the characterization of five halobacterial isolates from a sebkha near Ain Salah in Algeria that were able to produce biosurfactants, whose production and partial characterization are described.

Materials and methods

Source of organisms, media and growth conditions

Halobacteria were enriched from 50 samples collected at 1-m intervals in the ponds located close to Ain Salah in Algeria. The bacterial isolates were routinely cultured in a standard medium containing (per liter) 125 g of NaCl, 160 g of $MgCl_2{\cdot}6HO_2$, 5.0 g of K_2SO_4, 0.1 g of $CaCl_2{\cdot}2H_2O$, 1.0 g of yeast extract (Difco), 1.0 g of casamino acids (Difco) and 2.0 g of soluble starch (BDH) [50]. The pH of the medium was adjusted to pH 7.0 with NaOH. This medium was modified with respect to salt concentrations and nutrients as described below. In most experiments, cells were grown in a horizontal shaking water bath (200 strokes per min) at 40°C in 100-ml Erlenmeyer flasks containing 50 ml of medium. To prepare agar plates, the media were solidified with 20 g of agar per liter. The media were sterilized by autoclaving. For further studies, the growth media was modified; it contained diesel oil (5% v/v) as the sole carbon source. Diesel used in the experiments was a standard diesel fuel, without additives, obtained directly from Naftal Oil Refinery in Algiers. The cultures were purified by repeatedly streaking them on solid medium. Typically, the isolates grew well after 7 days in the standard medium at 40°C, pH 7.0 with 3.5 M NaCl.

Morphological, biochemical and physiological characterization

Gram staining was performed by using acetic acid-fixed samples as described by Dussault [23]. Tests for catalase and oxidase activities, starch, gelatine, casein and Tween 80 hydrolysis, formation of indole from tryptophan and nitrate reduction were performed by using standard procedures [28]. Growth response to NaCl was examined in liquid standard medium using serial NaCl concentrations ranging from 50 to 350 g l^{-1} and to pH by testing growth at pH 5–10. The growth response to temperature was examined by testing growth in liquid medium up to 60°C. The requirement for Mg^{2+} for growth was tested qualitatively by growing the strains in standard liquid medium with and without $MgSO_4{\cdot}7H_2O$. The utilization of sugars (glucose, fructose, galactose, arabinose, raffinose, xylose, cellobiose, sucrose and rhamnose) and the acid production from these compounds were determined in standard medium modified as follows: starch was omitted, and the yeast extract and casamino acids concentrations were reduced to 0.25 g l^{-1} each or yeast

extract and casamino acids were omitted, as described below. In the latter case, the media were amended with 0.1 g of NH_4Cl per liter and 0.01 g of KH_2PO_4 per liter [50]. Each potential carbon source was added to a final concentration of 5 g l^{-1} from a concentrated sterile solution. Growth was monitored by determining the optical density of each culture at 600 nm, and the pH of each culture was compared with the pH of a control culture. A decrease in the pH to a value less than 6.0 was considered evidence of acid production. Starch hydrolysis was tested by flooding colonies grown on agar plates containing the standard growth medium with an iodine solution. Susceptibility to antibiotic penicillin G (10 U) was determined in liquid medium.

DNA extraction, polymerase chain reaction and sequencing 16S ribosomal DNA

Bacterial strains designated as A21 and D21 were selected for molecular identification. DNA was extracted from the polycarbonate filters as described by Minz et al. [47], then the DNA was electrophoresed for 30 min at 100 V on 1% TAE agarose gel, excised from the gel and purified with a jet sorb gel extraction kit (Genomic DNA purification system-PROM, EGA). Purified DNA from the various strains was amplified using specific 16S rRNA archaeal primers, (21f 5′-TTCCGGTTGATCCYGCCGGA-3′) and (958r 5′-YCCGGCGTTGAMTCCAATT-3′) [20]. Each 50 µl reaction mixture contained 5 µl of 10 × PCR buffer, 5 µl of deoxynucleoside-triphosphate mix (2.5 nM each), 2.5 µl of bovine serum albumin, 0.5 µl of 21f primer (50 µM), 0.5 µl of 958r primer (50 µM), 0.5 µl of Taq polymerase (TaKaRa, Otsushiga, Japan), 1 µl of template DNA and RNase/DNase-free water to a final volume of 50 µl. PCR was performed in 50-µl glass capillaries using a Perkin-Elmer 480 thermal cycler. The following PCR program was used: 94°C for 30 s, followed by 30 cycles of 94°C for 15 s, 55°C for 20 s and 72°C for 45 s, followed by 72°C for 30 s. Phylogenetic analysis was performed using the software package BioNumerics (Applied Maths, Belgium) after including the sequence as received in an alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library EMBL. This alignment was pairwise calculated using an open gap penalty of 100% and a unit gap penalty of 0%. Similarity matrix was created by homology calculation with a gap penalty of 0% and after discarding unknown bases. A resulting tree was constructed.

Screening for biosurfactant-producing strains

The most important surface-active properties evaluated in screening microorganisms with potential industrial application are surface tension (ST) reduction, the emulsion-forming and -stabilizing capacity. The criterion used for selecting biosurfactant producers is the ability to reduce the ST below 40 mN m^{-1} [9, 16], whereas a criterion cited for emulsion-stabilizing capacity is the ability to maintain at least 50% of the original emulsion volume 24 h after formation [59]. Strains were cultivated on the standard medium, and screening of biosurfactant-producing colonies was performed using the qualitative drop-collapse test described by Jain et al. [35] after being modified by Bodour and Maier [9]. Motor oil, corn oil and olive oil also were evaluated for use in this test. Two microliters of oil was applied to the well regions delimited on the covers of 96-well microplates (Biolog, Hayward, CA) and left to equilibrate for 24 h. Five microliters of the 7th day strain cultures was transferred to the oil-coated well regions after centrifugation at 12,000 × *g* for 5 min to remove cells. Drop size was observed 1 min later with the aid of a magnifying glass; a result was considered positive for biosurfactant production when the drop diameter was at least 1 mm larger than that produced by sterilized standard medium (negative control).

Emulsification activity assay and surface tension measurement

Isolates testing positive in the drop-collapse test were also evaluated for emulsion-forming and -stabilizing capacity, according to the method proposed by Das et al. [21]; the ST of the cell-free supernatant was determined [51].

After growing in standard for 7 days in an orbital shaker at 160 rpm and 40°C, cells were removed by centrifugation at 12,000 × *g* for 5 min at room temperature. Two milliliters of the cell-free supernatant was mixed with 2 ml kerosene in a test tube (100 mm × 15 mm). This mixture was shaken for 2 min and then left to stand. Relative emulsion volume (EV, %) and emulsion stability (ES, %) were measured in intervals up to 48 h using the following equations [21]:

$$\mathrm{EV},\ \% = \frac{\text{emulsion height (mm)} \times \text{cross-section area (mm}^2\text{)}}{\text{total liquid volume (mm}^3\text{)}} \times 100$$

$$\%\mathrm{ES} = \frac{\%\mathrm{EV}, \%\ \text{at time } t, \text{h}}{\mathrm{EV},\ \%\ \text{at 0 h}} \times 100$$

Emulsions formed by the isolates were compared to those formed by a 1% (w/v) solution of the synthetic surfactant sodium dodecyl sulfate in deionized water, as proposed by Das et al. [21]. During growing in standard medium, the ST of the cell-free supernatant (50 ml) collected at different time intervals after centrifugation (4,500×*g*) for 10 min was determined using a KRUSS F6 tensiometer following the Wilhelmy plate measurement technique at room temperature [51].

Properties of emulsions

Stabilization of emulsions from halophilic strain was evaluated over a range of chemical and physical conditions. The extract material of the crude biosurfactant was dissolved in distilled water, and the pH was adjusted between 2 and 11 with HCl or KOH. After diesel oil was added, tubes were vortexed, and the emulsions were measured after 1 h. The emulsifier was tested with 10, 15, 20, 25 and 35% (w/v) sodium chloride and 0, 10, 25 and 50% (v/v) ethanol in the aqueous phase. For the evaluation of stability, emulsions containing 0.14% (w/v) purified biosurfactant in distilled water and diesel oil were incubated at 4°C and room temperature for an extended period. Emulsions were subjected to three cycles of heating (40°C, 16 h) and cooling (room temperature, 8 h) [13]. The stability of the formed emulsions (ES, %) was measured in intervals up to 48 h [21].

Surfactant isolation and purification

A crude biosurfactant preparation was obtained by centrifuging (10,000×*g*, 10 min, 4°C) the stationary-phase culture to remove the cells and adjusting the pH of the spent medium to 2 with 1 N HCl [17]. The acidified liquid was kept at 4°C overnight, and the precipitate that formed was collected by centrifugation (17,300×*g*, 30 min, 4°C). The precipitate was dissolved in distilled water; the pH was adjusted to 7.0 with 1 N NaOH, freeze dried and weighed. The lyophilized material was extracted three times with chloroform/methanol (2:1, v/v) solvent system. The extract was dried with the aid of a rotary evaporator under vacuum [32]. The method used for biosurfactant purification was modified from the work of Kim et al. [39]. Instead of the column chromatography steps used by Kim et al. [39], further purification was achieved by preparative thin-layer chromatography (TLC) of the extract. The extract was dissolved in distilled water (250 µl) and spotted onto preparative silica gel TLC plates (Whatman, Clifton, NJ) with a solvent system of chloroform/methanol (2:1, v/v). The components were observed under UV light (wavelength of 280 nm). Each fraction was scraped off the plate, dissolved in 250 µl of water and tested for surface activities using the qualitative drop-collapse test. Surface-active fractions were lyophilized.

The extract material was analyzed by TLC [1]. All organic solvents used were commercially distilled and of the highest available purity (Sigma-Aldrich). Plates for TLC (Silica gel 60A), obtained from Merck, were washed twice with chloroform/methanol (1:1, v/v) and activated at 120°C before use. Glucids and peptidic components were separated in solvent S1 (chloroform–methanol–acetic acid, 80:18:2 by volume). The peptidic components were visualized by staining them with ninhydrin (5 mg of ninhydrin in a 50 ml butanol–50 ml acetone mixture) and heating them at 100°C for 5 min [34]. Sugar compounds were located by charring at 110°C for 5 min after spraying anthrone reagent [34]. The solvent system S2 (chloroform–methanol–acetic acid, 97:2:1 by volume) was used for lipid migration. The lipid components were detected as brown spots on the plate after spraying with chromosulfuric acid [1].

Results

Characterization of isolates

The enrichment procedure we used selects mainly halobacteria. Colonies from the enrichments that developed on solid media were about 1 mm in diameter, circular, entire and pigmented red-orange after 1 week of incubation at 40°C.

The strains had salt growth requirements that were always above 15% (w/v) salts with an optimal concentration of 15–25% and were considered extremely halophilic. These strains were presumptively identified as members of the family Halobacteriaceae on the basis of phenotypic characteristics as shown in Table 1. The strains A21 and D21 were gram negative, motile, catalase and oxidase positive. On the basis of the phenotypic features tested, strains A21 and D21 showed phenotypics features resembling members of the genera *Halovivax* [15] and *Haloarcula*, respectively. The phylogenetic position of strains A21 and D21 are shown in Fig. 1; for the first strain, designated A21, the partial gene sequence obtained was 400 nucleotides in length (GenBank, AM982815); for the second strain, designated D21, the partial gene sequence obtained was 900 nucleotides in length (GenBank, AM982816). The sequences are comparable to 16S rRNA of other halophilic archaeon. We have demonstrated that strain A21 possessed similarities higher than 97% with those of the genus *Halovivax*. A similarity (based on a very small partial sequence) significant for possible species relatedness (>97%) is found with the two validly described *Halovivax* spp., thus indicating that the strain A21 approaches the genus *Halovivax* to a great extent. Also, the partial 16S rRNA sequence of strain D21 was determined (900 bp); the sequence was compared with the published 16S rRNA sequences of representative members of the Archaea. The sequence showing a significant similarity (97%) for possible species relatedness is found with several validly described *Haloarcula* spp. Seeing the distance matrix indicated that strain D21 may belong to one of these species. The following analyses for further species identification are suggested by the complete 16S rDNA sequence analysis and DNA: DNA hybridizations.

Table 1 Characteristics that distinguish strain A21 and strain D21 from other related haloarchaeal genera: *Halovivax* and *Haloarcula*

Characteristics	Strain A21	*Halovivax*	Strain D21	*Haloarcula*
Morphology	Coccus/pleomorphic	Rod/pleomorphic rod	Coccus/pleomorphic	Pleomorphic rod
Gram	−	−	−	−
Pigmentation	Pink-orange	Pale-pink	Pink-red	Red
Catalase and oxydase	+	+	+	+
NaCl optimum (M)	2.7	3.4	2.7	2.5–4.3
NaCl range (M)	2.7–4.5	2.5–4.3	2.7–4.5	1.7–5.2
Mg^{++} optimum (M)	0.8	0.05–0.1	0.5	0.05–0.1
pH Optimum	7–9, opt. 7	6–9, opt. 7–7.5	6–7.5, opt. 7	6.5–7.5
Temperature optimum (°C)	35–40	25–45, opt. 37	35–50	35–53
Nitrite from nitrate	−	−	−	+
Acid from carbohydrate	+	V	+	+
Growth on single carbon source	+	−	+	+
Indole from tryptophan	+	−	+	V
Hydrolysis of:				
Starch	+	−	+	V
Gelatin	+	+	+	V
Casein	+	+	+	−
Tween 80	+	+	+	V
Lysis in distilled water	Cells lyse	Cells lyse	Cells lyse	Cells lyse
Susceptibility to penicillin G (10 U)	−	+	−	+

Data from Castillo et al. [15] and this study

+ positive, − negative, *V* variable

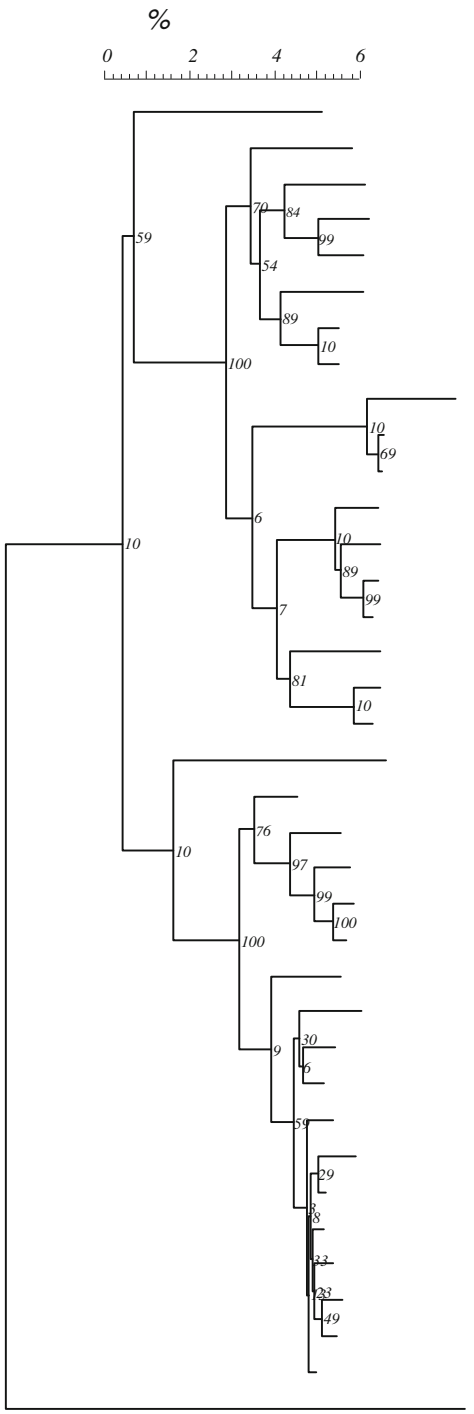
Screening and kinetic analysis of biosurfactant production

Five bacteria strains (A21, B21, C21, D21 and E21) were selected for their ability to grow at neutral pH and at 3–5.2 M NaCl and were screened for biosurfactant production and emulsification activity. They tested positive for biosurfactants in the drop-collapse test reduced as shown as Table 2. In this study the drop-collapse technique was only applied as a qualitative method to detect biosurfactant production. Motor oil proved better to work with than olive oil since it caused spreading of the sterilized standard medium used as negative control and produced plates in which drop diameter was most readily estimated, an important factor given this test is based on visual observation.

According to Bodour and Maier [9], the criterion used for selecting biosurfactant producers is the ability to reduce the ST below 40 mN m^{-1}. Also, in Willumsen and Karlson [59], a criterion cited for emulsion-stabilizing capacity is the ability to maintain at least 50% of the original EV 24 h after formation. The two strains tested produced extracellular biosurfactant and produced a strong biosurfactant capable of generating a stable emulsion over several hours. However, cell-free supernatant from the five isolates (A21, B21, C21, D21 and E21) exhibited reduced ST as shown in Table 2. Biosurfactants were either adhered to, or an integral part of, the cell surface of isolates that only reduced the ST in the presence of cells.

The relative emulsion volume (EV, %) was highest (75.2%) in the culture of the strain D21 followed by the strain A21 with the EV, % equal at 72.3%. In comparison, growth and ST decreased during 9 days of these five halophilics strains. The ST of the standard medium had a straight decreasing during the stationary growth phase (shown in Fig. 2a, b). All of the strains tested show the same kinetics of growth; we can see clearly that the production of biosurfactant takes place during the lag phase. The shapes of the curves are explained by a maximum production of the biosurfactants during this phase of the growth. They act as a primary metabolite. However, it is clear that strains D21 and A21 exhibited the highest surface activity with the lowest ST of 26.20 and 28.40 mN m^{-1}, respectively.

We saw the behavior of the strains A21 and D21 in the absence of a hydrocarbon in the culture medium. Thus, for the latter studies, the growth media was modified containing diesel oil (5% v/v) as the sole carbon source. The kinetics of growth was observed and shown in Fig. 3a, b. We note that the pH remains practically unchanged during the fermentation time of strains A21 and D21. The lower surface activity is marked



JCM 8858T	*Natronomonas pharaonis*	*D87971*
NCIMB 2189T	*Natronobacterium gregoryi*	*D87970*
FERM P-11024T	*Natronococcus amylolyticus*	*D43628*
DSM 18795T	*Natronococcus jeotgali*	*EF077631*
NCMB 2192T	*Natronococcus occultus*	*Z28378*
C231T	*Halobiforma nitratireducens*	*AB045012*
JCM 11627T	*Halobiforma haloterrestris*	*AF333760*
JCM 12983T	*Halobiforma lacisalsi*	*AY277582*
Souche A21		
DSM 18193T	*Halovivax ruber*	*AM269467*
CECT 7098T	*Halovivax asiaticus*	*AM039978*
JCM 10478T	*Natrinema versiforme*	*AB023426*
JCM 12890T	*Natrinema altunense*	*AY208972*
NCIMB 786T	*Natrinema pellirubrum*	*AJ002947*
NCIMB 784	*Natrinema pallidum*	*AJ002948*
JCM 10989T	*Natrialba hulunbeirensis*	*AF262026*
ATCC 700177T	*Natrialba asiatica*	*D14123*
DSM 13077T	*Natrialba aegyptia*	*AF251941*
ATCC 700874T	*Halomicrobium mukohataei*	*D50850*
ATCC 33960T	*Haloarcula hispanica rrnB*	*AB090168*
ATCC 49778T	*Haloarcula japonica*	*D28872*
ATCC 700850T	*Haloarcula quadrata rrnB*	*AB010965*
JCM 13557T	*Haloarcula amylolytica rrnA*	*DQ826512*
ATCC 43049T	*Haloarcula marismortui rrnA*	*X61688*
ATCC 33800	*"Haloarcula sinaiiensis" minor gene*	*D14130*
NBRC 14741T	*Haloarcula vallismortis*	*D50851*
"B-2"	*"Haloarcula aidinensis"*	*AB000563*
JCM 13557T	*Haloarcula amylolytica rrnB*	*DQ826513*
JCM 13557T	*Haloarcula amylolytica rrnC*	*DQ854818*
Souche D21		
ATCC 33960T	*Haloarcula hispanica rrnA*	*AB090167*
ATCC 700850T	*Haloarcula quadrata rrnA*	*AB010964*
ATCC 43049T	*Haloarcula marismortui rrnB*	*X61689*
ATCC 33800	*"Haloarcula sinaiiensis" major gene*	*D14129*
ATCC 29715T	*Haloarcula vallismortis*	*U17593*
JCM 9737T	*Haloarcula argentinensis*	*D50849*
DSM 864T	*Methanospirillum hungatei*	*M60880*

Fig. 1 Phylogenetic dendrogram of halobacteria based on 16S rRNA gene sequence data, indicating the position of the strains A21 and D21. The tree was constructed using the neighbor-joining method. The sequence data used for the following strains were obtained from the sequences collected from the international nucleotide sequence library EMBL. *Methanospirillum hungatei* was used as outgroup. Alignment was pairwise calculated using an open gap penalty of 100% and a unit gap penalty of 0%. Similarity matrix was created by homology calculation with a gap penalty of 0% and after discarding unknown bases

for the strain D21. ST is the force required to break the surface between two immiscible phases. However, we note that the ST in the 1st days of fermentation is quite high, but at the end of the 3rd day, we see a decline in values until they reach 28 and 34 mN m^{-1} for the two bacterial strains A21 and D21, respectively. As for the pH of the culture medium, it remained stable and stationary all along microbial growth. This result allows us to say that there is a

Table 2 Surface tension of culture media without cells and relative volume of emulsions formed between cell culture media and diesel oil after growth of bacteria strains in standard medium for 7 days at 40°C and 200 rpm

Strains	Drop-collapse	Surface tension	Relative emulsion volume (%) after 48 h Cell-free supernatant
A21	++++	28.4 ± 1.2	72.3 ± 0.6
B21	+++	36.4 ± 0.6	65.8 ± 0.7
C21	+++	35.5 ± 1.4	66.9 ± 0.5
D21	++++	26.2 ± 0.8	75.2 ± 0.4
E21	+++	36.9 ± 0.6	66.3 ± 0.6
1% SDS	++++	42.8 ± 0.6	23.5 ± 0.8

Surface tension was expressed as mN m^{-1} using standard medium as control (73.9 mN m^{-1}); values reported are average of 6–12 replicates. %EV was as percentage

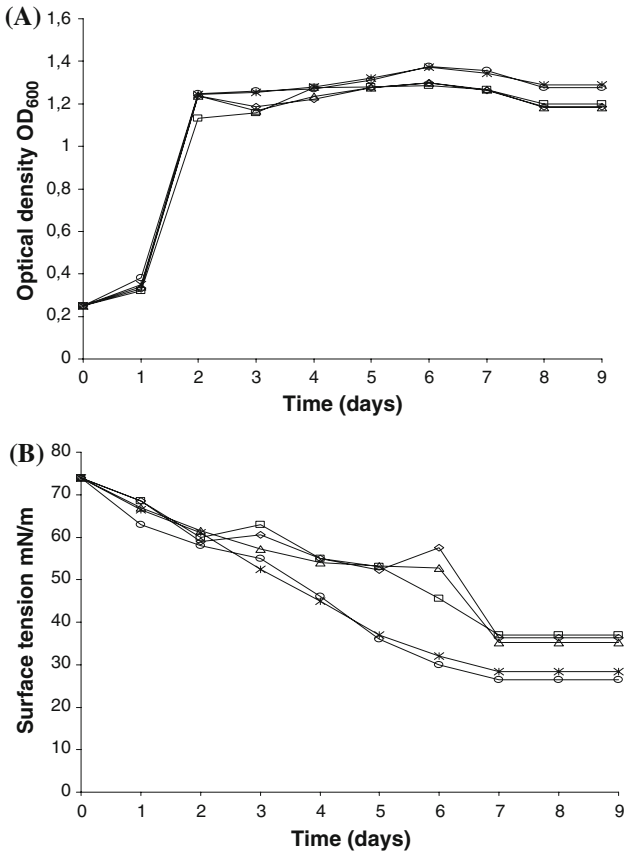


Fig. 2 Growth (**a**) and surface tension (**b**) decrease by cultures of five halophilic strains (A21 *asterisk*, B21 *open diamond*, C21 *open triangle*, D21 *open circle* and E21 *open square*). Each culture of these strains was grown at 40°C and 200 rpm in standard medium with soluble starch (BDH) as a carbon source. Values are averages for three cultures

production of biomolecules with surface activity as biosurfactants that cause the reduction of the ST of the medium. In addition and according to these results, it is

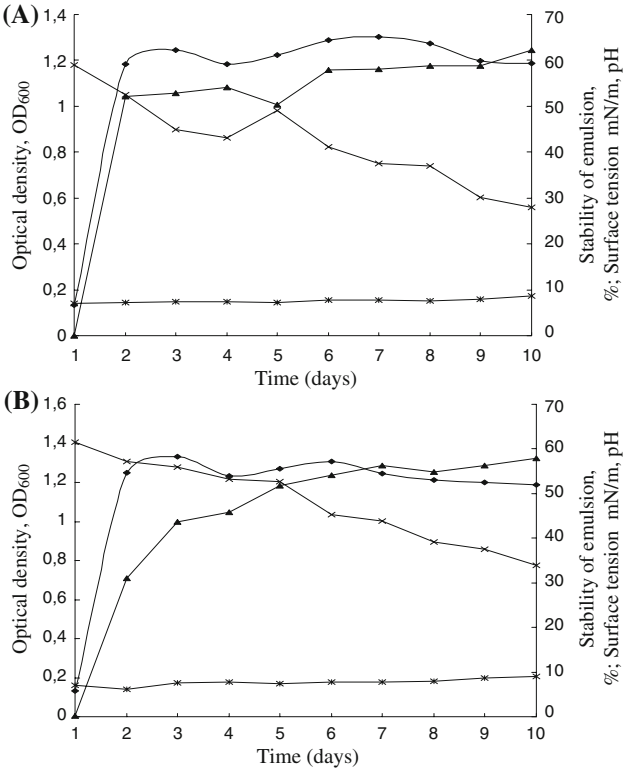


Fig. 3 Kinetic growth (*filled diamond*), emulsion stability (*filled triangle*), pH (*asterisk*) and ST(*multiplication sign*) decreases by cultures of strain A21 (**a**) and strain D21 (**b**). Each culture of these strains was grown at 40°C and 200 rpm in medium with diesel oil (5% v/v) as a carbon source. Values are averages for three cultures

clear that these extremely halophilic bacteria grow on a medium containing hydrocarbons in a similar way to that in the presence of the starch. The influence of NaCl concentration on the kinetics of the production of biosurfactant was tested over the range of 0–5 M (Fig. 4a, b). The kinetics of biosurfactant included the variation of emulsion stability and ST decreases by cultures of the strains A21 and D21. There was no production in the absence of added NaCl. The production increased with salt concentration up to 2 M NaCl, and then decreased again at 5 M NaCl. Thus, the strains A21 and D21 exhibited the highest surface activity with the lowest ST of 27.4 and 25.90 mN m^{-1}, respectively. This result clearly indicates that production of biosurfactants is salt dependent.

Chromatographic behavior

Strain A21 presented lipids with a regular mobility in the solvent S2 (RF, 0.34), and the lipids gave a negative test on TLC plates for strain D21. Protein and sugar gave a positive test on TLC plates for both of strains A21 and D21, indicating that there is a production of the extracellular compounds by the strains A21 and D21. According to these

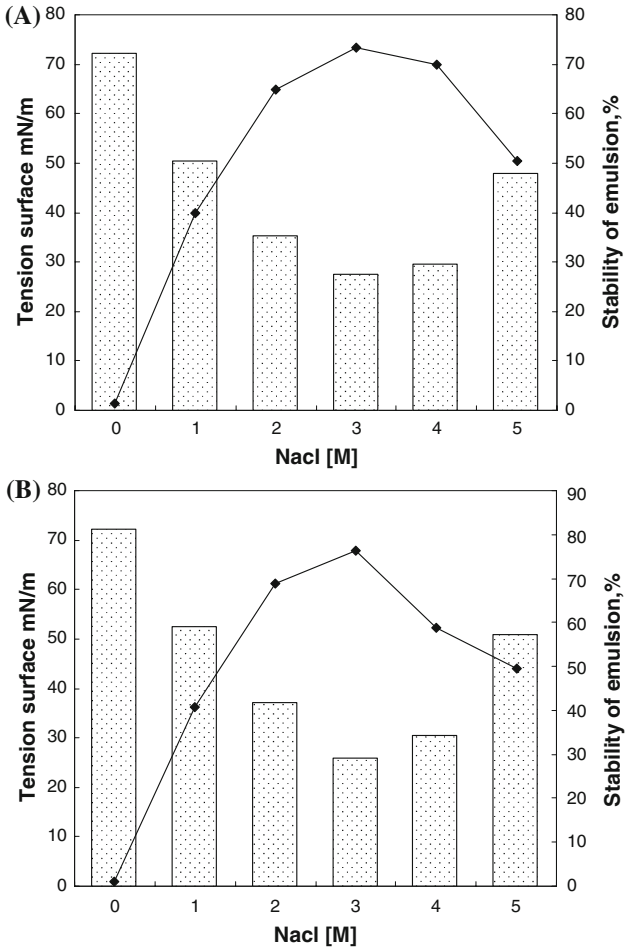


Fig. 4 Effect of NaCl concentrations on the kinetic of biosurfactant: variation of emulsion stability (*filled diamond*) and surface tension (*dot filled box*) decreases by cultures of strain A21 (**a**) and the strain D21 (**b**). Each culture of these strains was grown at 40°C and 200 rpm in standard medium. Values are averages for three cultures

results, the biosurfactants produced by the halophilic bacteria D21 could be glycoproteins, whereas for the halophilic strain A21, the components of the biosurfactant were found to contain sugar, protein and lipid. This could thus be a peptidoglycolipid, such as glycoprotein, glycolipid or lipopeptide.

Properties of the emulsions

Strains A21 and D21 were selected to study the stability of the emulsions formed under various conditions. The emulsions were tested for stabilization under a range of chemical and physical conditions that might be encountered in various applications. According to Cameron et al. [13], it clearly facilitates the detection of the possible detrimental effects of pH, sodium chloride or ethanol on emulsification. Emulsions from both strains, A21 and D21, were made with 0.05% (w/v) purified crude biosurfactant.

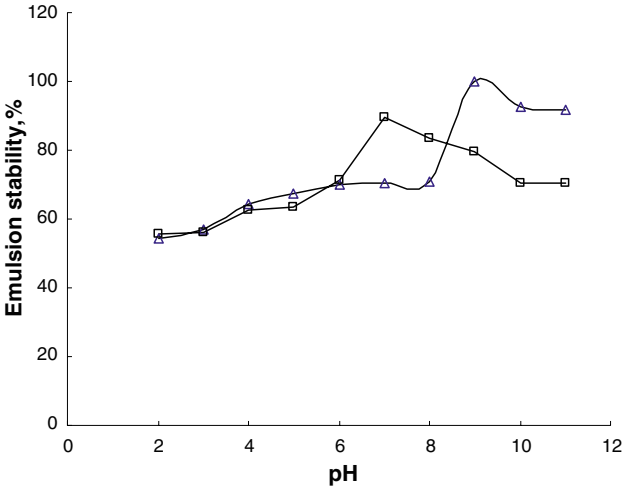


Fig. 5 Influence of pH on the stability of the emulsion formed by the strains (A21 *open triangle* and D21 *open square*) after diesel oil was added. The emulsions were measured after 1 h of rest at room temperature

The dilution by distilled water indicates that it does not have an effect on the emulsion produced. The emulsion-stabilizing capacity of the two bacterial strains A21 and D21 is kept constant with a value of 60.2 and 58.82%, respectively. Figure 5 shows that the pH of the aqueous phase had little effect on the amount of diesel oil phase emulsified between pH 2 and 11. In the basic environments, it is clear that the strain A21 has an emulsion-stabilizing capacity that is more important than those in the neutral and acidic environments, with an optimum at pH 9. After 48 h at room temperature, the relative emulsion stability is equal to 100%. The strain D21 shows the same result except that its highest emulsion-stabilizing capacity appears with neutral pH. The effect of the change in pH does not appear to affect the emulsions formed. These results demonstrate once again that it is possible to use these, fermenting in sites that are polluted, for example, by oil, whatever the pH values of the site where clean up is needed. As shown in Fig. 6, in the presence of 10–35% (w/v) sodium chloride in the aqueous phase, stable and strong emulsions were formed of both strains A21 and D21. According to the results obtained, strains A21 and D21 showed that the relative emulsion stability formed increases with increasing concentrations of ethanol until it reaches a maximum at a concentration of 25% (ES % = 93%). However, a higher concentration in ethanol (50%) caused a decrease in the relative emulsion stability (ES % = 88%). Figure 7 illustrates that the presence of high concentrations of ethanol has a positive effect on the stability of the emulsion. The cycle of temperature (40, 25, 4, −4°C) has no effect on the stability of emulsions formed. But the temperature cycle in reverse (−4, 4, 25 to 40°C) causes the dispersion of the emulsion, so this physical treatment provokes reduced emulsion stability.

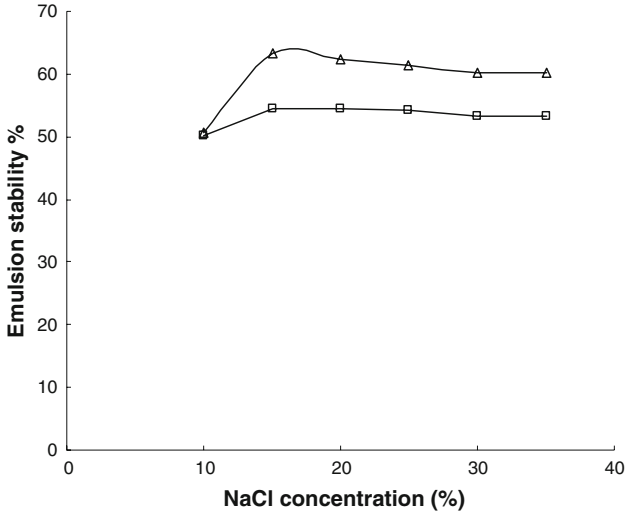


Fig. 6 Effect of different concentrations of salt (NaCl) on the stability of the emulsion formed by strain A21 (*open triangle*) and strain D21 (*open square*). The emulsions were measured after 48 h of rest at room temperature

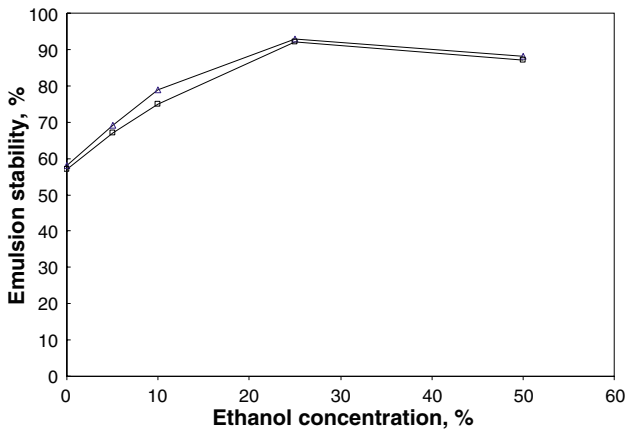


Fig. 7 Effect of different concentrations of ethanol (%) on the stability of the emulsion formed by strain A21 (*open triangle*) and strain D21 (*open square*). The emulsions were measured after 48 h of rest at room temperature

Emulsions formed around the strains A21 and D21 were not disrupted by three cycles of heating to 40°C (16 h) and then cooling to 23°C (8 h). During storage at 4°C, the relative emulsion stability did not change over a 4-month period.

Discussion

This study broadens the field of biosurfactant-producing microorganisms. Given the potential of biosurfactants to be used as tools in different fields, our work suggests that it is interesting to search for biosurfactant in an extremely halophilic archaeon. Certainly there is current interest in the production of other biosurfactants from halophiles, both archaeal and eubacterial, given the possibility that biosurfactants adapted to high salt concentrations and temperatures will have improved stabilities when used in organic solvents. In this study, the screening for biosurfactant production under hypersaline conditions is described for archaeal strains. Five Halobacteria strains were used throughout this work, two of which, A21 and D21, are identified as better biosurfactant producers using the qualitative drop-collapse test and the emulsification activity assay. They were selected for identification by phenotypic, biochemical characteristics and by partial 16S rRNA gene sequencing. We demonstrated that they are able to grow in the presence of 25% NaCl. Analyses of partial 16S rRNA gene sequences of the two strains suggested that they were extremes halophiles belonging to genera of the family Halobacteriaceae; the strain A21 is very close to the genus *Halovivax*, and the strain D21 could be the genus *Haloarcula*. Also, halophiles have usually been overlooked in most screening programs for exopolymers. Recently, it has been found that *Haloferax mediterranei* produces a highly sulfated and acidic heteropolysaccharide (up to 3 g l^{-1}), which contains mannose as a major component [3, 4]. Such a polymer combines excellent rheological properties with a remarkable resistance to extremes of salinity, temperature and pH [3]. Sulfated EPS is also notable for its role in inhibiting viral penetration into cells [33]. *Haloferax mediterranei* and *Haloarcula japonica* both produce sulfated EPS [6, 33]. The partial characterization of biosurfactants produced by strains A21 and D21 is the primary focus of the study. According to the results obtained, the biosurfactants produced by the halophilic bacteria D21 could be glycoproteins, whereas for the halophilic strain A21, the components of the biosurfactants were found to contain sugar, protein and lipid. This thus could be a peptidoglycolipid, such as glycoprotein, glycolipid or lipopeptide. It is currently known that the type of biosurfactant made is dictated by the producing microorganism. One major class of biosurfactants is the glycolipids, which includes rhamnolipids, trehalose lipids and sophorose lipids. Rhamnolipids are produced only by *P. aeruginosa* [10]; trehalose lipids are produced only by a number of closely related genera, including *Rhodococcus*, *Nocardia*, *Corynebacterium*, *Tsukamurella*, *Gordonia*, *Mycobacterium* and *Arthrobacter* [10] and other glycolipids produced by *Streptococcus thermophilus* [55]. Polymeric biosurfactant producers have been isolated from Eubacteria, Eukaryotes and Archaea [10]. Moreover, the halobacterial membrane lipids exhibit many relevant properties; the ether-linked lipids possess very low melting points, are resistant to degradation by acids, alkalis and heat, and have an emulsifying ability, with an adequate hydrophile-lipophile balance, which produces good water-in oil emulsions [52]. An interesting potential

application of the unique ether-linked lipids of the halobacteria is their use in novel types of liposomes, which have great value in the cosmetic industry. Such liposomes would be more resistant to biodegradation than those used at the moment and thus would have a better shelf life, since halobacterial lipids are relatively resistant to the action of other bacteria [27]. Salt concentration also affected biosurfactant production depending on its effect on cellular activity. Some biosurfactant products, however, were not affected by salt concentrations up to 10% (w/v), although slight reductions in the critical micelle concentration were detected [48]. Furthermore, growth was not inhibited by the presence of crude oil. The biosurfactant produced was not affected by the temperatures, pHs or NaCl. In this study, we observed that strains A21 and D21 were capable of reducing the surface tension of the medium up to 28 mN m^{-1} or more. Therefore, our own values are in agreement with values obtained by other researchers [31, 44], which are around 19–28 mN m^{-1}. The biosurfactant production is directly proportional to cell growth. When cell growth increases, ST decreases. The ST became constant and increased slightly during the late stationary phase. Similar findings were also obtained for other surfactant-producing eubacteria [5, 18]. Cell-free supernatant from all halophilics strains tested in this study exhibited reduced ST. Biosurfactants were either adhered to, or an integral part of, the cell surface of isolates that only reduced the ST in the presence of cells [16]. Isolates that liberate biosurfactants into the culture medium are interesting from an industrial point of view, because the product recovery process can be simplified [12, 26, 41]. The biopolymers secreted by halophiles are intrinsically highly stable and may have applications as mobility controllers and emulsifying agents in the oil industry [3, 5]. There are very few reports on hydrocarbon biodegradation in hypersaline environments. Ward and Brock [58] have shown an inverse relationship between hydrocarbon biodegradation and salinity. Bertrand et al. [8] reported the isolation of halophilic hydrocarbonoclastic bacteria, showing that hydrocarbon metabolism may occur in hypersaline conditions. In our studies, starch was a better carbon source than the other carbohydrates for screening biosurfactant-producing strains. Studies showed that few halophilic Archaea are able to grow in aromatic compounds, but the production of biosurfactants was never demonstrated [19]. *Haloferax volcanii* D1227, a halophilic archaeon isolated from oil-brine-contaminated soil, was shown to degrade mono-aromatic compounds, such as benzoate, cinnamate and 3-phenylpropionate [24]. More recently, *Haloarcula sp. D1* was shown to metabolize *p*-hydroxybenzoic acid [25]. Furthermore, the potential application of Halobacteria (halophilic Archaea) for bioremediation of recalcitrant compounds in highly saline wastewaters for the accelerated remediation of hydrocarbon-polluted saline environments has been considered [7, 42]. The biological treatment of high saline effluents, such as the waters produced from the oil industry, has been studied [2]. Both selected strains are being identified and quantification and physicochemical characterization of their surface-active metabolites completed as the basis for optimization studies aimed at application in bioremediation.

Conclusion

Five Halobacteria strains were used throughout this work for research on the production of biosurfactants under hypersaline conditions. Two of the strains, A21 and D21, are identified as better biosurfactant producers using the qualitative drop-collapse test and the emulsification activity assay. The strains have Mg^{2+} and salt growth requirements that are always above 15% (w/v) salts with an optimal concentration of 15–25%. Analyses of partial 16S rRNA gene sequences of the two strains suggested that they were halophilic Archaea belonging to genera of the family Halobacteriaceae. Strain D21 is very close to the genus *Haloarcula*, and strain A21 approaches the genus *Halovivax*. These two strains showed the highest surface activity with the lowest ST of 26.20 and 28.40 mN m^{-1}, respectively. Also, these extremely halophilic bacteria grow on a medium containing hydrocarbons in a similar way as in the presence of the starch. The biochemical nature of the biosurfactants produced by these halobacteria thus could be a peptidoglycolipid, such as glycoprotein, glycolipid or lipopeptide. The high stability of the emulsions formed under various conditions may be useful in various industries. Thus, there is an increasing interest in the possible use of these biosurfactants in mobilizing heavy crude oil, oil pollution control, cleaning oil sludge from oil storage facilities, oil/sand bioremediation and microbially enhancing oil recovery.

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